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This report describes methods for the measurement of the acute toxicity of effluents to macroinvertebrates and fish. The methods include a preliminary short-term (8-24 hr), range-finding (screening) test and a long-term (96 hr) flow-through, or alternate static, definitive test for use in determining the LC50 or EC50 of the waste. The report includes guidelines for effluent sampling and holding, facilities and equipment, dilution water, test species selection and handling, and data interpretation.

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METHODS FOR MEASURING THE ACUTE TOXICITY OF EFFLUENTS TO AQUATIC ORGANISMS

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FOREWORD

Environmental measurements are required to determine the quality of ambient water, the character of effluents, and the effects of pollutants on aquatic life. The Environmental Monitoring and Support Laboratory-Cincinnati conducts research to develop, evaluate, and promulgate methods to:

- Measure the presence and concentration of physical, chemical and radiological pollutants in water, wastewater, bottom sediments, and solid waste.
- Concentrate, recover, and identify enteric viruses, bacteria, and other microorganisms in water.
- Measure the effects of pollution on freshwater, estuarine, and marine organisms, including the phytoplankton, zooplankton, periphyton, macrophyton, macroinvertebrates, and fish.
- Automate the measurement of the physical, chemical, and biological quality of water.
- Conduct an Agency-wide quality assurance program to assure standardization and quality control of systems for monitoring water and wastewater.

The Federal Water Pollution Control Act Amendments (Clean Water Act) of 1977 (PL 95-217) explicitly state that it is the national policy that the discharge of toxic substances in toxic amounts be prohibited. Determination of the toxicity of effluents, therefore, has high priority in the EPA water pollution control program. However, suitable, standardized methodology for effluent bioassays has not been available to EPA regional and state programs. This report fills an urgent current need for standardized methods to measure the toxicity of effluents to aquatic life.

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PREFACE

The effluent toxicity tests described in this document were prepared by the Bioassay Subcommittee of the EPA Biological Advisory Committee to provide methods needed for effluent monitoring by EPA Regional and State NPDES programs and for self-monitoring by NPDES permit holders. Two types of toxicity tests are described:

1. A preliminary, short-term, static, range-finding (screening) test for use in determining the concentrations of effluent to be used in a "definitive" test.

2. A long-term (generally 96-hr), flow-through, definitive test for use in determining the acute toxicity of the effluent, expressed as a LC50 or EC50.

These methods will be included in the second edition of the EPA manual, "Biological Field and Laboratory Methods for Measuring the Quality of Surface Waters and Effluents," but were printed separately in limited quantity to make them available prior to the publication of the manual.

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ABSTRACT

This report describes methods for the measurement of the acute toxicity of effluents to macroinvertebrates and fish. The methods include a preliminary short-term (8-24 hr), range-finding (screening) test and a long term (96 hr.), flow-through (or alternate static) definitive test for use in determining the LC50 or EC50 of the effluent. The report includes guidelines on effluent sampling and holding, facilities and equipment, dilution water, test species selection and handling, and data interpretation.

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SECTION 1

INTRODUCTION

The Declaration of Goals and Policy of the Federal Water Pollution Control Act Amendments (Clean Water Act) of 1977 (PL 95-217), Section 101(a)(3), states that "it is the national goal that the discharge of toxic pollutants in toxic amounts be prohibited". Current Agency programs for the protection of aquatic life in receiving waters are based in part on effluent limitations for individual chemicals. However, toxicity data are available for only a limited number of compounds. Effluent limitations, therefore, may not provide adequate protection where the toxicity of the components in the effluent is not known, where there are synergistic effects between toxic substances in complex effluents, and/or where a complete chemical characterization of the effluent has not been carried out. Since it is not economically feasible to determine the toxicity of each of the thousands of potentially toxic substances in complex effluents or to conduct exhaustive chemical analyses of effluents, the most direct and cost-effective approach to the measurement of the toxicity of effluents is to conduct a bioassay with aquatic organisms representative of indigenous organisms in receiving waters. For this reason, the frequency of use of effluent bioassays to identify and control toxic discharges is rapidly increasing within the Agency and state NPDES programs, and by permittees as a self-monitoring requirement.

The lack of standardized bioassay methodology developed explicitly for effluents has delayed the implementation of EPA regional and state effluent toxicity testing programs and has resulted in a lack of uniformity in test procedures. In response to this problem, a subcommittee was organized within the EPA Aquatic Biology Methods Advisory Committee, sponsored by the Environmental Monitoring and Support Laboratory, Cincinnati, to prepare effluent bioassay methods for the NPDES program. To provide valid methods, it was essential to include EPA Regional and Enforcement programs personnel with extensive experience in conducting effluent toxicity tests. In completing their task, the subcommittee members drew from their own experience and borrowed heavily from the report, "Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians," (EPA 660/3-75-009), previously prepared primarily to standardize basic laboratory methods for determining the toxicity of pure compounds.

The acute toxicity tests for effluents described in this report are used to determine the effluent concentration, expressed as a percent volume, that is lethal to, or has some other "adverse effect" on, 50 percent of the organisms within 96 hrs or some other prescribed period of time. If mortality (death) is the effect measured, the toxicity is expressed as the median lethal concentration or LC50. Where death is not easily detected, for example, with some invertebrates, other indicators such as immobilization must be used as the adverse effect. Blood chemistry, biochemical measurements or histological examinations can also be employed to determine the toxicity of an effluent. The concentration of effluent, expressed as a percent volume, that causes a defined adverse effect other than death in 50 percent of the test organisms within the prescribed exposure period is termed the median effective concentration or EC50.

A two-test sequence is generally required to estimate the acute toxicity of an effluent: (1) a preliminary, short-term (8-24 hr), range-finding test is conducted to define the range of effluent dilutions to be used in the definitive test, and (2) a more rigorous long-term definitive test is conducted (using the range of effluent dilutions determined by the range-finding test) over a 96-hour time period to arrive at the acute toxicity of the effluent, which is expressed as a LC50 or EC50.

One of the following procedures shall be used for the range-finding test:

A. Test organisms are placed in suitable containers and exposed under static conditions to 3-5 widely-spaced dilutions of the effluent for a period of 8-24 hours.

B. If the effluent has a high dissolved oxygen demand, or the organisms require flowing water, a flow-through test is used to define the range of toxicity of the effluent.

One of the following procedures shall be used for the definitive test:

A. Preferred procedures (flow-through tests), in order of preference

1. Test organisms are exposed to effluent solutions flowing into and out of test chambers on a once-through basis for the duration of the test. The effluent is conveyed directly and continuously from the source to the dilutor system.

2. Test organisms are exposed to effluent solutions flowing into and out of test chambers on a once-through basis for the duration of the test as in A.1 above. However, the effluent is supplied to the dilutor system from discrete effluent samples collected periodically. The interval at which samples are collected is based on the variability of the effluent characteristics, production schedule, batch processes, retention time, etc. (see p. 17 - Effluent Sampling and Holding).

The continuous effluent sampling technique (A.1) is the best of two methods described.

B. Alternative procedures (static tests), in order of preference

1. Test organisms are exposed to a fresh solution of the same concentration of effluent every 24 hours, either by transferring the test organisms from one test chamber to another, or by replacing the effluent solution in the test chambers.

2. Test organisms are exposed to the same effluent solution for the duration of the test. However, the effluent solution in

each test chamber may be filtered, aerated, or sterlized by continuous circulation through an appropriate apparatus.

3. Test organisms are exposed to the original effluent solution for the duration of the test without being continuously circulated through an apparatus as in B.2.

The alternative procedures may be used in emergency situations or where adequate facilities are not available to the investigator. However, it must be understood that a test using the alternative procedures is not valid unless it can be conclusively demonstrated that the chemical characteristics and toxicity of the effluent do not change over time. Because of toxicant adsorption on the test chambers, uptake by test organisms and the effect of metabolites on toxicity, it is preferred that the effluent solution be renewed at least once every 24 hours as described in B.1 above.

The special environmental requirements of some organisms, such as flowing water, fluctuating water levels, or substrate may preclude the use of static testing.

SECTION 2

FACILITIES AND EQUIPMENT

GENERAL REQUIREMENTS

Effluent toxicity tests may be carried out in a fixed or mobile lab. Depending upon the scope of the bioassay program, facilities may include equipment for rearing, holding and acclimating organisms. Temperature control is achieved using circulating water baths, or environmental chambers. Dilution water may be ground water, surface water, reconstituted water, or dechlorinated tap water. Holding, acclimation, and dilution water should be temperature-controlled and aerated whenever possible. Air used for aeration must be free of oil and fumes; filters to remove oil in water are desirable. Test facilities must be well ventilated and free of fumes. During holding, acclimating, and testing, test organisms should be shielded from disturbances.

Some organisms may have special environmental requirements such as flowing water, fluctuating water levels, or substrate which must be provided. During holding, acclimating, and testing, immature stream insects should always be in flowing water; as described by Nebeker and Lemke (1968); penaeid shrimp and bottom-dwelling fish should be provided a silica sand substrate. Since cannibalism can occur among many species of arthropods, they should be isolated by some means (e.g., with screened compartments), or the claws of crabs and crayfish should be bound.

CONSTRUCTION MATERIALS

Materials used in the construction of the test equipment which come in contact with the effluent should be carefully chosen. Glass, No. 316 stainless steel, and perfluorocarbon plastics (TEFLON^{R}) should be used whenever possible to minimize leaching, dissolution, and sorption. Linear polyethylene may also be used with some types of industrial and municipal effluents, but should be avoided with those containing synthetic organic compounds or pesticides. Unplasticized plastics such as polyethylene, polypropylene, TYGON^{R} and fiberglass can be used for holding, acclimating, and dilution-water storage tanks, and in the water delivery system. Copper, galvanized material, rubber, brass, and lead must not come in contact with holding, acclimation, or dilution water, or with effluent samples and test solutions.

EFFLUENT (TOXICANT) DELIVERY SYSTEM (Flow-through test only)

Although many types of toxicant delivery systems have been designed*, the flow-through, proportional-dilutor delivery system has proven to be the best and the preferred system for routine effluent toxicity tests conducted in both fixed and mobile laboratories.

The following factors should be considered in designing the system: (1) whether the apparatus will be installed and used in a fixed or mobile laboratory; (2) the existence of adequate space and/or structural requirements for the delivery system, test chambers, effluent and dilution-water storage; (3) the applicability of the delivery system to specific effluent characteristics (high suspended solids, volatiles, etc.); (4) the system's dependability, durability, flexibility, and ease of maintenance and replacement; (5) the ability to perform within the flow rate and concentration accuracy limitations; and (6) the cost of the system.

Two types of proportional dilutors are described in the appendix - the solenoid valve system and the vacuum siphon system. The solenoid valve system is preferred, but the vacuum siphon system is acceptable, if funds are limited.

The flow rate through the proportional dilutor must provide for at least five complete water volume changes in 24 hours in each test chamber, plus sufficient flow to maintain an adequate concentration of dissolved oxygen. It is often desirable to construct the dilutor with an additional reserve flow capacity, depending on its application and/or special effluent characteristics. The flow rates through the test chambers should not vary by more than 10 percent among test chambers at any time during any test. The dilutor should also be capable of maintaining the test concentration in each test chamber within 5 percent of the starting concentration for the duration of the test.

The calibration of the dilutor should be checked carefully before and after each test. This check should determine the volume of effluent and dilution water used in each portion of the effluent delivery system and the flow rate through each test chamber. The general operation of the dilutor should be checked at least at the beginning and end of each day during the test.

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*(Lowe, 1964; Mount and Brungs, 1967; Sprague, 1969; Freeman, 1971; Cline and Post, 1972; Granmo and Kollberg, 1972; Bengtsson, 1972; Lichatowich, et al., 1973; Schumway and Palensky, 1973; Abram, 1973; Schimmel, Hansen, and Forester, 1974; DeFoe, 1975; Riley, 1975).

TEST CHAMBERS

Type

Test chambers used in flow-through tests are constructed of 1/4 inch plate glass held together with (GE) clear silicone adhesive. Silicone adhesive absorbs some organochlorine and organophosphorus pesticides, which are then difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water; extra beads of adhesive inside the containers should be removed. Stainless steel (#316) can be used in the construction of test chambers, but must be of welded, not soldered, construction.

The size of the chambers may vary according to the size of the test organism and/or the facilities, but the test solution should have a minimum depth of 5 cm. All chambers should have either a glass or screen cover to prevent organisms from jumping out.

The test chambers most commonly used in static tests are wide-mouth, 3.9 liter (1-gallon) or 19.6 liter (5-gallon) soft-glass bottles. Containers such as 1-liter battery jars or 250-ml beakers may be more suitable as test chambers for fish eggs and/or larvae and small crustacea. Special glass or stainless steel test chambers can be constructed to accommodate test organisms requiring special physical conditions.

Cleaning

All test chambers, whether new or used, must be washed in the following manner to remove surface contaminants:

A. Soak and wash in suitable detergent in water, preferably heated to a temperature of 50°C or higher. The detergent (powder or liquid) should be entirely synthetic (SPARKLEEN® or ALCONOX®).

B. Rinse with water (preferably heated to 50°C or higher).

C. Rinse with a fresh, dilute (5 percent) hydrochloric acid for removing metals and bases.

D. Rinse with water (preferably heated to 50°C or higher).

E. Rinse with acetone to remove organic compounds. When contaminated with a pesticide, test chambers must be rinsed with acetone before they are placed in the hot detergent soak (Item A. above).

F. Rinse twice with water.

When feasible, the outlined cleaning procedure must be used for other equipment that comes in contact with the dilutor system, pumps, tanks, etc. All test chambers and equipment must be thoroughly rinsed with the dilution water prior to each test.

SECTION 3

TEST ORGANISMS

SPECIES

Whenever possible, effluent toxicity tests should be conducted with a sensitive species that is indigenous to the receiving water, readily available, and either commercially or recreationally important. Acceptable species include those listed in Table 1, which are sensitive to most toxicants. The test organisms must be identified to species.

SOURCE

Although effluent tests should be conducted with species that are indigenous to, or stocked into, the receiving water, the test organisms do not have to be taken from the receiving water. It is often difficult to obtain fish of the desired size and condition from the receiving water. Collection permits are often difficult to obtain, and the organisms in the receiving water may have been previously exposed to the effluent. Fish captured by electroshocking must not be used in testing. The usual sources of freshwater fish used for toxicity tests are private, state, and Federal hatcheries. If trout are used as test organisms, they should be obtained from stock that has been certified as disease-free.

Some fish, such as Fathead and Sheepshead minnows, are easily reared under laboratory conditions (USEPA, 1972; Schimmel and Hansen, 1974). However, it is usually more practical to collect marine species from indigenous populations.

Some invertebrates such as daphnids, midges, and shrimp may be reared in laboratory cultures. Care must be taken to insure that only young age groups and early instars are used in testing. Daphnids from cultures in which ephippia are being produced should not be used in the tests. Invertebrates not amenable to laboratory rearing are usually obtained directly from wild populations.

SIZE

Very immature fish (not actively feeding on exogenous food), spawning fish, or recently spent fish should not be used. Fish weighing between 0.5 and 5.0 grams each are preferred. In any single test, all fish should be taken from the same year class, and the total length (tip of snout to end of tail) of the longest fish should be no more than 1-1/2 times that of the shortest one. As stated above, immature invertebrates should be used whenever possible.

TABLE 1. RECOMMENDED SPECIES AND TEST TEMPERATURES

Species	Test Temperature (°C)[a]
Freshwater	
Vertebrates	
Coho salmon, Oncorhynchus kisutch	12
Rainbow trout, Salmo gairdneri	12
Brook trout, Salvelinus fontinalis	12
Goldfish, Carassius auratus	22
Fathead minnow, Pimephales promelas	22
Channel catfish, Ictalurus punctatus	22
Bluegill, Lepomis macrochirus	22
Invertebrates[a]	
Daphnids, Daphnia magna or D. pulex	17
Amphipods, Gammarus lacustris, G. fasciatus, or G. pseudolimnaeus	17
	17
Crayfish, Orconectes sp., Cambarus sp., Procambarus sp., or Pacifastacus leniusculus	22
	22
Stoneflies, Pteronarcys sp.	12
Mayflies, Baetis sp. or Ephemerella sp.	17
Hexagenia limbata or H. bilineata	22
Midges, Chironomus sp.	22
Marine and estuarine	
Vertebrates	
Sheepshead minnow, Cyprinodon variegatus	22
Mummichog, Fundulus heteroclitus	22
Longnose killifish, Fundulus similis	22
Silverside, Menidia sp.	22
Threespine stickleback, Casterosteus aculeatus	22
Pinfish, Lagodon rhomboides	22
Spot, Leiostomus xanthurus	22
Shiner perch, Cymatogaster aggregata	12
Pacific staghorn sculpin, Leptocottus armatus	12
Sanddab, Citharichthys stigmaeus	12
Flounder, Paralichthys dentatus, P. lethostigma	22
English sole, Parophrys vetulus	12

TABLE 1. (Cont'd)

Species	Test Temperature (°C)[a]
Marine and estuarine Invertebrates[a]	
Shrimp, *Penaeus setiferus*, *P. duorarum*, or *P. aztecus*	22
Grass shrimp, *Palaemonetes* sp.	22
Shrimp, *Crangon* sp.	22
Oceanic shrimp, *Pandalus jordani*	12
Blue crab, *Callinectes sapidus*	22
Dungeness crab, *Cancer magister*	12
Mysid shrimp, *Mysidopsis* sp., *Neomysis* sp.	22
Atlantic oyster, *Crassostrea virginica*	22
Pacific oyster, *Crassostrea gigas*	20

[a]Freshwater amphipods, daphnids, and midge larvae and shrimp should be cultured and tested at the recommended test temperature. Other invertebrates should be held and tested within 5°C of the temperature of the water from which they were obtained. If the recommended test temperature is not within this range, they should be tested at the temperature from the series 7, 12, 17, 22, and 27°C that is closest to the recommended test temperature and is within the allowed range.

HOLDING AND HANDLING

Disinfect holding tanks or chambers with 0.5 percent commercial bleach for one hour. Brush thoroughly with the disinfectant. Rinse well between groups of organisms. Other equipment used to handle organisms must be disinfected with 0.5 percent commercial bleach (5 ml of bleach added to 1 liter of water), or 30-percent formalin.

When feasible, holding tanks must receive an uncontaminated, constant-quality water in a flow-through system with a flow rate of at least 2 tank-volumes per day. Otherwise, a recirculation system where the water flows through a charcoal filter to remove dissolved metabolites or passes by an ultra-violet light for disinfection is necessary. Only as a last resort should a dechlorinated tap water be used for freshwater organisms or a synthetic salt water for marine organisms.

When organisms are first brought into a facility, they must be quarantined for a minimum of 10 days. To avoid unnecessary stress after collection and transportation, organisms should not be subjected to a change of more than a 3°C in water temperature or 3-ppt in salinity in any 12-hour period, or a total change of not more than 6°C or 6 ppt salinity. Invertebrates should be held within 5°C of the temperature of the water from which they were obtained. To maintain organisms in good condition during holding, crowding should be avoided. The dissolved oxygen concentration must be greater than 40 percent of saturation for warm water species and greater than 60 percent of saturation for cold water species. Aeration may be used if necessary.

Organisms should be fed at least once a day, and excess food and fecal material should be removed from the bottom of the tanks at least twice a week by siphoning. Organisms should be observed carefully for signs of disease, stress, physical damage, and mortality. Dead and abnormal specimens should be removed as soon as observed. A daily log of feeding, behavioral observations, and mortality must be maintained.

Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible to minimize stress. Organisms that touch dry surfaces or are dropped or injured during handling must be discarded. Dipnets are best for handling larger organisms. Such nets are commercially available or can be made from small-mesh nylon netting, nylon or silk bolting cloth, plankton netting, or similar material. Nets coated with urethane resin are best for handling catfish. Smooth glass tubes with rubber bulbs should be used for transferring smaller organisms such as daphnids and midge larvae.

DISEASE TREATMENT

During holding, fresh and salt water fish should be chemically treated to cure or prevent disease as recommended in Table 2. However, if the fish are severely diseased, it is advisable to discard the entire lot. When invertebrates become diseased, they should be discarded. Tanks which are contaminated with disease-causing microorganisms must be disinfected with 0.5 percent commercial bleach.

TABLE 2. RECOMMENDED PROPHYLACTIC AND THERAPEUTIC TREATMENTS FOR FRESHWATER FISH[a]

Disease	Chemical	Concentration (mg/l)	Duration of Treatment[c]
External Bacteria	Oxytetracycline hydrochloride (water soluble)	25[b]	30-60 min
	Procaine Penicillin G in Dihydrostreptomycin sulfate solution (Franklin Lab, Denver,CO)	(3ml/100 gal)	48-72 hrs
	Benzalkonium chloride (HYAMINE 1622[R])	1-2[b]	30-60 min
	Nitrofurazone (water mix)	3-5[b]	30-60 min
	Neomycin sulfate	25	30-60 min
Monogenetic trematodes fungi, and external protozoa[d]	Formalin plus zinc-free malachite green oxalate	25 0.1	1-2 hrs
	Formalin	150-250	30-60 min
	Potassium permanganate	2-6	30-60 min
	Sodium chloride	15000-30000 2000-4000	5-10 min dip (e)
	DEXON[R] (35% Active Ingredient)	20	30-60 min
Parasitic copepods	Trichlorfon (MASOTEN[R])	0.25[b]	Continuous[f]

[a]This table indicates the order of preference of treatments that have been reported to be effective, but their efficacy against diseases and toxicity to fish may be altered by temperature or water quality. Caution: test treatments on small lots of fish before making large-scale applications. Fish should not be treated the first day they are in the facility.

TABLE 2 (continued).

Before using a treatment other than those listed in this table, additional information should be obtained from sources such as Davis (1953), Hoffman and Meyer (1974), Reichenbach-Klinke and Elkan (1965), Snieszko (1970), and van Duijn (1973).

b. Active ingredient.

c. Treatment may be accomplished by (1) transferring the fish to a static treatment tank and back to a holding tank; (2) temporarily stopping the flow in a flow-through system, treating the fish in a static manner, and then resuming the flow to flush out the chemical; or (3) continuously adding a stock solution of the chemical to a flow-through system by means of a metered flow or the technique of Brungs and Mount (1967).

d. One treatment is usually sufficient except for Ichthyophthirius ("Ich"), which must be treated daily or every other day until no sign of the protozoan remains. This may take 4-5 weeks at 5-10°C and 11-13 days at 15-21°C. A temperature of 32°C is lethal to "Ich" in one week.

e. Minimum of 24 hours, but may be continued indefinitely.

f. Continuous treatment should be employed in static or flow-through systems until no copepods remain, except that treatment should not be continued for more than 4 weeks and should not be used above 27°C.

TRANSPORTATION AND ACCLIMATION

Organisms reared and held at a fixed facility are transported to the test site in the water in which they were reared and held. If the laboratory is mobile, the acclimation tank can be used in transporting organisms from the rearing and holding facilities to the test site. At the test site, dilution water (receiving water) is pumped to the laboratory for use in the acclimation of the organisms. If dilution water is not readily accessible, it can be transported to the laboratory and stored in a tank for use in the acclimation procedure and toxicity test. During transport and acclimation the organisms should not be subjected to a change of more than 3°C in water temperature or 3-ppt in salinity in any 12-hour period, or a total change of not more than 6°C or 6-ppt salinity, and the concentration of dissolved oxygen must not fall below 40 percent of saturation for warm water species and 60 percent of saturation for cold water species.

Upon arriving at the test site, the organisms are acclimated to the test dilution water and temperature by gradually changing from 100-percent holding water to 100-percent dilution water over a period of 24 hours. All organisms must be exposed to 100-percent dilution water for at least 24 hours before they are used for the tests, and must be held at the test temperature ($\pm$2°C) for at least 24 hours before tests are begun.

A group of organisms must not be used for a test if they appear to be diseased or otherwise stressed, or if more than 5 percent die during the 48 hours immediately preceding the test. If the organisms fail to meet these criteria, the entire group must be discarded and a new group obtained. The same acclimation procedure must be followed for the new group or ogranisms. Mortality of the test organisms during the acclimation period may be due to the presence of toxicants in the dilution water (receiving water). If excessive mortality occurs during the acclimation of the second group of test organisms, an alternate source of dilution water must be used.

The acclimation of marine organism for effluent toxicity tests poses special problems because most effluents discharged into the marine environment consist of adulterated freshwater. Therefore, when the effluent is diluted with the receiving water (salt water), the higher percent effluent volumes will have a low salinity (the salinity will be inversely proportional to the percent volume of effluent). If the effluent is essentially freshwater, it is obvious that 100% effluent cannot be used with the marine test organisms. The highest effluent concentration (lowest salinity) tested will depend upon the salinity of the receiving water and the tolerance of the test organism. (Sheepshead minnows and mysid shrimp are known to be tolerant to a salinity range of 5-35 ppt, but the tolerance of other marine species in Table 1 must be established by the investigator). Under the circumstances described above, it will be necessary to acclimate marine organisms to a series of salinities, ranging from 5-35 ppt. It would also be advisable to culture the test organisms at a series of salinity levels, including at least 10, 20, and 30 ppt, so that changes in salinity upon acclimation do not exceed 6 ppt.

SECTION 4

DILUTION WATER

A dilution water is acceptable if healthy test organisms survive in it through the acclimation period and toxicity test without showing signs of stress, such as discoloration or unusual behavior. For effluent toxicity testing, the dilution water should be a representative sample of the receiving water, and should be obtained from a point as close as possible to, but upstream of or outside of, the zone influenced by the effluent. It is preferable to pump the dilution water continuously to the acclimation tank and dilutor. However, it may be more practical to transport batches of water in tanks to the testing site as the need arises, and then continuously pump water to these systems.

In an estuarine environment, the investigator should collect uncontaminated water having a salinity as near as possible to the salinity of the water at the receiving site.

Pretreatment of the dilution water should be limited to filtration through a nylon sieve having 2-mm or larger holes to remove debris and/or break up large floating or suspended solids. The water should be obtained from the receiving water as close as possible to the time the test begins. It should not be obtained more than 96 hours prior to testing. If acceptable dilution water cannot be obtained from the receiving water, some other uncontaminated, well-aerated surface or ground water, or commercially available media, can be used. This water must have a total hardness, total alkalinity, and specific conductance within 25 percent, and pH within 0.2 units, of the receiving water at the time of testing.

If the substitute dilution water must be modified, reconstituted water must be prepared for use as the diluent. Recommended procedures are given in Tables 3 and 4. There are also commercially available salt water media such as INSTANT OCEAN^{R} and RILA SALTS^{R}.

With highly toxic effluents requiring very large volumes of dilution water, it may be convenient to locate the testing facility near the source of the dilution water, and transport the effluent.

TABLE 3. PREPARATION OF RECONSTITUTED FRESH WATERS [a]

3a. Quantities (mg/l) of reagent grade chemicals required to prepare recommended reconstituted fresh waters and the resulting water qualities.

Water Type	Reagent Added				Final Water Quality		
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCL	pH[b]	Hardness[c]	Alkalinity[c]
Very soft	12	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Hard	192	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384	240.0	240.0	16.0	8.0-8.4	280-320	225-245

3b. Quantities of reagent-grade chemicals to be added to aerated, soft reconstituted freshwater for buffering pH. The solutions should not be aerated after addition of these chemicals.

pH[d]	Volume (ml) of solution added to 15 liters of water		
	1.0N NaOH	1.0 m KH_2PO_4	0.5 m H_3BO_3
6.0	1.3	80.0	-----
6.5	5.0	30.0	-----
7.0	19.0	30.0	-----
7.5	---	----	-----
8.0	19.0	20.0	-----
8.5	6.5	----	40.0
9.0	8.8	----	30.0
9.5	11.0	----	20.0
10.0	16.0	----	18.0

a. From Marking and Dawson (1973).
b. Approximate equilibrium pH after aeration and with fish in water.
c. Expressed in mg/l as $CaCO_3$.
d. Approximate equilibrium pH with fish in water.

TABLE 4. PREPARATION OF RECONSTITUTED SEA WATER[a,b]

Add the following reagent-grade chemicals in the amounts and order listed to 890 ml distilled water. Each chemical must be dissolved before another is added.

Chemical	Amount
NaF	3 mg
$SrCl_2 \cdot 6H_2O$	20 mg
H_3BO_3	30 mg
KBr	100 mg
KCl	700 mg
$CaCl_2 \cdot 2H_2O$	1.47 g
Na_2SO_4	4.00 g
$MgCl_2 \cdot 6H_2O$	10.78 g
NaCl	23.50 g
$Na_2SiO_3 \cdot 9H_2O$	20 mg
Na_4EDTA(c)	1 mg
$NaHCO_3$	200 mg

a. If the resulting solution is diluted to 1 liter, the salinity should be 34±0.5 g/kg, and the pH 8.0±0.2. The desired salinity is attained by dilution at time of use.

b. From Kester et al. (1967), Zaroogian et al. (1969), and Zillioux et al. (1973).

c. Tetrasodium ethylenediaminetetraacetate. This coumpound should be omitted when toxicity tests are conducted with metals. When tests are conducted with plankton or larvae, the EDTA should be omitted and the medium should be stripped of trace metals (Davey et al., 1970).

SECTION 5

EFFLUENT SAMPLING AND HOLDING

SAMPLING

The effluent sampling point must be the same as that specified in the NPDES discharge permit. Conditions for exception would be: (1) better access to a sampling point between the final treatment and the discharge outfall, or (2) if the processed waste is chlorinated prior to discharge to the receiving waters, the sampling point may be located prior to contact with the chlorine if the purpose of the test is to determine toxicity levels of the unchlorinated effluent. Sampling should be based on an understanding of the short and long-term operations and schedules of the discharger. It is desirable to evaluate an effluent sample that most closely represents the "normal" or "typical" discharge and operating conditions of the plant in question. The retention time of the effluent in the waste water treatment facility, as indicated in Par. A.1.a-c and B.1-3 below, must be measured using dye studies. The only way in which the sample may be altered prior to testing is by filtering through a TEFLON® or stainless steel screen with 2-mm or larger holes.

A. Flow-through test

1. If the industrial or municipal facility discharges continuously, the effluent should be pumped directly and continuously from the discharge line to the dilutor system for the duration of the test. The use of the effluent grab samples should be avoided. However, if the effluent cannot be pumped directly and continuously to the dilutor system, the following alternative methods may be employed for collection of the effluent:

   a. When the measured minimum retention time of the effluent is less than 96 hours, as determined from dye studies, a 6-hour composite sample, consisting of equal volumes taken every 30 minutes, must be collected and transported to the dilutor every 6 hours for the duration of the test.

   b. When the measured minimum retention time of the effluent is between 4 days (96 hours) and 14 days, as determined from dye studies, then a 24-hour composite sample, consisting of equal volumes taken every hour, may be collected daily for the duration of the test.

c. When the measured minimum retention time of the effluent is greater than 14 days, as determined from dye studies, a single grab sample may be collected daily for the duration of the test.

2. If the industrial or municipal facility discharges intermittently (i.e. where the waste is discharged over a single 8-hr work shift, or is accumulated and discharged at the end of the shift, or end of the week), a composite sample, consisting of equal volumes collected every 30 minutes, may be taken for an 8-hr operating shift or for the duration of the plant operating schedule, or a single grab sample may be taken in the case of a batch discharge.

B. Static test

If a flow-through test cannot be used, a static test may be conducted with effluent collected by one of the following methods:

1. When the measured minimum retention time of the effluent is less than 96 hours, as determined with dye studies, four consecutive 6-hr composite samples, each consisting of equal volumes taken every 30 minutes, are collected and used in setting up 4 separate static tests.

2. When the measured minimum retention time of the effluent is between 4 days (96 hours) and 14 days, as determined with dye studies, a 24-hour composite sample, consisting of equal volumes taken every hour, is collected daily and used in the test.

3. When the measured minimum retention time of the effluent is greater than 14 days, as determined with dye studies, a single grab sample may be collected and used in the test.

HOLDING

Effluent grab samples must be stored in covered, unsealed containers. Violent agitation must be avoided. However, undissolved materials must be uniformly dispersed by gentle agitation. This agitation must immediately precede adjustment of any aliquot of the effluent to test temperatures before adding it to the dilution water. Although it is desirable to refrigerate samples prior to the test, it is often convenient to store samples in a constant-temperature water bath or controlled-environment room at the temperature at which the test is conducted. The test should be initiated as soon as possible, but no later than 24 hours after collection of the effluent.

The persistence of the toxicity of an effluent may be a factor in determining specific toxicity limits in an NPDES permit, and is determined by measuring its toxicity upon collection and again after holding 96 hours. If after holding the effluent 96 hours, its toxicity has not decreased 50% or more, it is classified as persistent. (When special tests, such as persistence are conducted, the exact methodology must be detailed in the report.)

SECTION 6

TEST PROCEDURE

RANGE-FINDING(SCREENING) TEST

Unless the approximate toxicity of the effluent is already known, it is necessary to conduct an abbreviated, preliminary, range-finding or screening test to determine the concentrations that should be used in the definitive tests. This test can be either a static or flow-through test. However, the test most often used is an abbreviated static test in which groups of 5 organisms are exposed to three to five widely-spaced effluent dilutions, and a control, for 8 to 24 hours.

Because the characteristics of the effluent and the receiving water may vary significantly within short periods of time, the toxicity observed in a range-finding test may not be representative of the toxicity of the effluent. If the range-finding test is to be conducted with the same sample of the effluent with which the definitive test is to be conducted, the duration of the range-finding test cannot exceed 24 hours (see limits in holding time for effluents, p. 18).

DEFINITIVE TEST

Test Conditions

The determination of a LC50 or EC50 must employ a control and at least five concentrations of effluent in an exponential series. To calculate the LC50 or EC50 with reasonable accuracy, a definitive test must meet both of the following criteria:

A. Each concentration of the effluent must be at least 50 percent of the preceding concentration.

B. One concentration must have killed (or affected) more than 65 percent of the organisms exposed to it, and one concentration other than the control must have killed (or affected) less than 35 percent of the organisms.

If 100-percent effluent does not kill (or affect) more than 65 percent of the organisms exposed to it, the percentage of organisms killed (or affected) by various levels of the effluent in the receiving water must be reported.

The control shall consist of the same dilution water, conditions, procedures, and organisms used in testing the effluent. A test is not acceptable if more than ten percent of the organisms die in the control.

Number of Test Organisms

At least 20 organisms of a given species must be exposed to each treatment. More than one species may be used in the same test chamber in a

given test, if segregated. One-half of the organisms of each species exposed to each treatment should be placed in separate test chambers to serve as replicates. To qualify as true replicates, no water connections can exist between replicate test chambers. Randomization of treatments is desirable.

Test animals are normally captured for transfer from acclimation tanks to test chambers by dip netting. No more than 20 percent of the total number of organisms transferred to each chamber should be added from a given net capture.

Loading of Test Organisms

For all tests, a limit must be placed on the weight of organisms per liter of test solution. This practice will minimize the depletion of dissolved oxygen, the metabolic conversion of effluent constituents, the accumulation of metabolic waste products, and/or stress induced by crowding, any of which could significantly affect the test results.

For flow-through tests, loading in the test chambers must not exceed 5 grams per liter at temperatures of 20°C or less, or 2.5 grams per liter at temperatures above 20°C.

For static tests, loading in the test chambers must not exceed 0.8 grams per liter at temperatures of 20°C or less and 0.4 grams per liter at temperatures above 20°C.

Test Temperature

For flow-through tests, it is desirable to hold the temperature within $\pm$2.0°C of the acclimation temperature throughout the test. This can be accomplished by passing the effluent and/or dilution water through separate stainless steel coils immersed in a heating or cooling water bath prior to entering the dilutor system.

For static tests, the temperature may be that at which the test organisms were held prior to transportation or acclimation at the site. The instantaneous ambient temperature should not vary more than $\pm$2°C at any time during the test.

Dissolved Oxygen

Aeration may alter the results of toxicity tests and, as a general rule, should not be employed. It can reduce the apparent toxicity of an effluent by stripping it of highly volatile toxic substances, or increase its toxicity by altering the pH. However, the dissolved oxygen concentration (DO) in the test solution should not be permitted to fall below 40 percent saturation for warm water species and 60 percent saturation for cold water species. In most flow-through tests, DO depletion is not a problem in the test chambers because aeration occurs as the liquids pass through the dilutor system.

If the DO concentration decreases to a level that would be a source of additional stress, the turnover rate of the solutions in the test chambers must be increased sufficiently to maintain acceptable DO levels. If the increased turnover rate does not maintain adequate DO levels, aerate the dilution water prior to the addition of the effluent, and aerate all test solutions.

Caution must be exercised to avoid excessive aeration, and it should be used only as a last resort in maintaining adequate DO levels. When aeration is used, the exact methodology must be detailed in the report.

Beginning the Test

The test begins when the test organisms are first exposed to the effluent.

A. Flow-through test

The dilutor system should be in operation 24 hours prior to the addition of the test organisms and the beginning of the test. During this period, necessary adjustments can be made in the percent effluent volumes, temperature, and flow rate through the test chambers.

B. Static test

The effluent is added to the dilution water and mixed well by stirring with a glass rod. The test organisms are placed in the chambers within 30 minutes. This procedure conserves DO and is sufficient for the effluent to become evenly dispersed in the dilution water.

Feeding

Organisms should not be fed during the test unless they are newly hatched or very young. In the case of fish, feeding should be terminated 48 hrs before the beginning of the test. Problems caused by feeding, such as the possible alteration of toxicant concentration, the build-up of food and metabolic wastes and resulting oxygen demand, are common in static test systems, but are minimal in flow-through systems.

Duration

The test duration may range from a minimum of 8 hours to 96 hours, depending on the test organism used, the purpose of the test and whether it is a range-finding test or a definitive test.

SECTION 7

TEST RESULTS

BIOLOGICAL DATA

The lengths and weights of the test organisms should be determined by sacrificing and measuring representative organisms before the test or by obtaining the lengths and weights of all surviving organisms at the end of the test. The number of dead (or affected) organisms in each test container should be counted 24, 48, 72, and 96 hours after the beginning of the test. (See data sheet in Appendix, Fig. 1, p. 33). Dead organisms must be removed at least once every 24 hours.

Death is the "effect" most frequently used for determining toxicity to aquatic organisms. The criteria usually employed in establishing death are: (1) no movement (especially no gill movement in fish), and (2) no reaction to gentle prodding. Death is not easily determined for some invertebrates, and some other effect often must be used. The effect generally used for determining toxicity to daphnids and midge larvae is immobilization, which is defined as the inability to move except for minor activity of appendages. With crabs, crayfish, and shrimp, the effects used are immobilization and loss of equilibrium. Other effects can be used for determining an EC50, but the effect and its definition must always be reported. General observations on such things as erratic swimming, loss of reflex, discoloration, changes in behavior, excessive mucus production, hyper-ventilation, opaque eyes, curved spine, hemorrhaging, molting, and cannibalism should be reported.

CHEMICAL AND PHYSICAL DATA

The dissolved oxygen concentration and pH must be measured at the beginning of the test, and every 24 hours thereafter, in the control and in the high, medium, and low effluent concentrations, for the duration of the test. The specific conductance, total alkalinity, total hardness, and salinity, where applicable, should be measured at the beginning of the test in the control and the high, medium and low effluent concentrations. There may be a build-up of ammonia in the static toxicity tests. It is advisable, therefore, to measure the concentration of total ammonia nitrogen in the control, high, medium, and low effluent concentrations at the beginning and end of each static test. The percentage of un-ionized ammonia in the test containers can be determined from Tables 5 and 6. Temperature should be recorded at least hourly in at least one container during the acclimation period and test.

TABLE 5. PERCENTAGE OF AMMONIA THAT IS UN-IONIZED IN DISTILLED WATER AT VARIOUS TEMPERATURES AND pH's[a]

PERCENT UN-IONIZED AMMONIA[b]									
Temperature	pH								
(°C)	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
7	0.01	0.05	0.15	0.46	1.45	4.44	12.8	31.7	59.5
12	0.02	0.07	0.22	0.68	2.13	6.44	17.9	40.8	68.5
17	0.03	0.10	0.32	1.00	3.08	9.14	24.1	50.1	76.1
22	0.04	0.14	0.45	1.43	4.39	12.7	31.5	59.2	82.1
27	0.06	0.21	0.65	2.03	6.15	17.2	39.6	67.4	86.8

TABLE 6. SALINITY CORRECTION FACTOR[c]

Salinity (g/kg)	Factor
5	0.82
10	0.80
15	0.77
20	0.75
25	0.73
30	0.72
35	0.72

a. Skarheim (1973), and Thurston et al.(1974).
b. These values can be corrected for salinity by multiplying by the appropriate factor from Table 6.
c. The resulting values should be within about ±5% over the range of pH from 6 to 9, and temperature from 0° to 30°C.

Water samples collected for chemical analysis should be taken midway between the top, bottom, and sides of the test containers and should not include any surface scum or material stirred up from the bottom or sides.

Methods used for chemical analysis must be those specified in Section 304(g) of the Federal Water Pollution Control Act Amendments of 1972 (Chemical Methods Manual, USEPA, 1977).

CALCULATION OF LC50 AND EC50

For each set of data, the 96-hr LC50 or EC50 and its 95-percent confidence limits must be calculated on the basis of the initial volume percent of the effluent in the test solutions. The "volume percent" equals "(100 x volume of effluent)/(volume of effluent + volume of dilution water)." If other (24-,48-,72-hr) LC and EC values are calculated, their 95 percent confidence limits must also be determined. A variety of methods are available to calculate a LC and EC (Finney, 1964, 1971). The most widely used are the log-concentration versus-percent-survival, probit, logit, moving-average, and Litchfield-Wilcoxon (1949) methods.

Two examples of calculating a LC50 using a hypothetical set of data have been provided in the Appendix. The two methods used in the calculations are: (1) the Litchfield-Wilcoxon and, (2) the log-concentration-versus-percent-survival. If more than 10 percent of the control organisms die, none of the previously mentioned methods may be used in calculating LC and EC values, and the remaining test results must be used with caution in evaluating the toxic effect.

REPORTS

A report of the results of a test must include the following:

A. The name of the test method, investigator and laboratory, and the date the test was conducted.

B. A detailed description of the effluent, including its source, date and time of collection, composition, known physical and chemical properties, and variability.

C. The source of the dilution water, the date and time of its collection, its chemical characteristics, and a description of any pretreatment.

D. Detailed information about the test organisms, including scientific name, length and weight, age, life stage, source, history, observed diseases, treatments, and acclimation procedure used.

E. A description of the test procedure: the test chambers, including the depth and volume of solution; the way the test was begun; the number of organisms per treatment; and the loading. For the flow-through system, the water volume changes per 24 hours in each test chamber must be calculated and reported.

F. The definition of the adverse effect (death, immobility, etc.) used in the test, and a summary of general observations on other effects or symptoms.

G. The number and percentage of organisms in each test chamber (including the control chambers) that died or showed the "effect" used to measure the toxicity of the effluent.

H. A 24-, 48-, 72-, and 96-hr LC50 or EC50 value for the test organisms, depending on the duration of exposure. If 100 percent effluent did not kill or affect more than 65 percent of the test organisms, report the percentage of the test organisms killed or affected by various concentrations of the effluent.

I. The 95-percent confidence limits for the LC50 and EC50 values and the method used to calculate them.

J. The methods used for and the results of all chemical analyses.

K. The average and range of the acclimation temperature and the test temperature.

L. Any deviation from this method.

M. Any other relevant information.

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34. Zillioux, E. J., H.R. Foulk, J.C. Prager, and J. A. Cardin. 1973. Using Artemia to assay oil dispersant toxicities. JWPCF. 45:2389-2396.

APPENDIX

A. LITCHFIELD AND WILCOXON ABBREVIATED METHOD OF DETERMINING THE LC50

General Procedure

Step 1: Tabulate the data (see sample data sheet, Fig. 1, p. 33) showing the percent-effluent volumes used, the total number of organisms exposed to each percent-effluent volume, the number of affected organisms, and the observed percent-affected organisms (see Example 1 below). Do not list more than 2 consecutive 100 percent affects at the higher percent-effluent volumes or more than two consecutive 0 percent affects at the lower percent-effluent volumes.

Step 2: Plot the percent-affected organisms against the percent-effluent volume on 2 cycle, logarithmic probability paper (Fig. 2), except for 0 percent or 100 percent affect values. With a straight edge, fit a temporary line through the points, particularly those in the region of 40 percent to 60 percent affects.

Step 3: Using the line drawn through the points, read and list an "expected" percent affect for each percent-effluent volume tested. Disregard the "expected" percent value for any of the percent volumes less than 0.01 or greater than 99.99. Using the expected-percent-affect, calculate from Table 7 a "corrected" value for each 0 percent or 100 percent affect obtained in the test. (Since the expected values in the table are whole numbers, it will be necessary to obtain intermediate values by interpolation.) Plot these values on the logarithmic probability paper (Fig. 2) used in Step 2 and inspect the fit of the line to the completely plotted data. If after plotting the corrected expected values for 0 percent and 100 percent affected, the fit is obviously unsatisfactory, redraw the line and obtain a new set of expected values.

Step 4: List the difference between each observed (or corrected) value and the corresponding expected value. Using each difference and the corresponding expected value, read and list the contributions to Chi-square (Chi^2) from Fig. 3 (a straight edge connecting a value on the Expected-Percent Affected scale with a value on the Observed-Minus-Expected scale, will indicate at the point of intersection of the Chi^2 scale, the contribution to Chi^2. Sum the contributions to Chi^2 and multiply the total by the average number of organisms per effluent volume, i.e., the number of organisms used in K concentrations divided by K, where K is the number of percent-affected organism values plotted. The product is the "calculated" Chi^2 of the line. The degrees of freedom (N) are 2 less than the number of points plotted, i.e., N =K-2. If the calculated Chi^2 is less than the Chi^2 given in Table 8 for N degrees of freedom, the data are non-heterogeneous and the line is a good fit. However, if the calculated Chi^2 is greater than the Chi^2 given in Table 8 for N degrees of freedom, the data are heterogeneous and the line is not a good fit. In the event a line cannot be fitted (the calculated Chi^2 is greater than the tabular Chi^2), the data can not be used to calculate a LC50 or EC50. Litchfield and Wilcoxon provided an alternate method for calculating the 95 percent confidence limits under these circumstances. However, the toxicity test should be repeated.

Step 5: Determine the confidence limits of the LC50.

a. Read from the fitted line (Fig. 2), the percent effluent volumes for the corresponding 16, 50, 84 percent affects (LC16, LC50 and LC84).
b. Calculate the slope function, S, as:

$$S = \frac{LC84/LC50 + LC50/LC16}{2}$$

c. From the tabulation of the data determine N', which is defined as the total number of test organisms used within the percent-affected-organism interval of 16 percent and 84 percent. Calculate the exponent $(2.77/\sqrt{N'})$ for the slope function and the factor, f_{LC50}, used to establish the confidence limits for the LC50 (or EC50).

$$f_{LC50} = S^{(2.77/\sqrt{N'})}$$

The f_{LC50} can be obtained directly from the nomogram in Fig.4 by laying a straight-edge across the appropriate base and exponent values and reading the resultant "f" value.

e. Calculate the confidence limits of the LC50 as follows:

(1) Upper limit for 95% probability = LC50 X f_{LC50}

(2) Lower limit for 95% probability = LC50/f_{LC50}

Example

Steps 1-4: The data were tabulated and plotted (Fig. 2) and the expected values were read from the graph.

STEP ONE				STEP THREE	STEP FOUR	
% Effluent Volume	Number of Organisms	Number of Affected Organisms	Observed % Affected Organisms	Expected % (Fig. 2)	Observed Minus Expected	Chi^2
3.2	20	0	0(.2)[b]	.6	0.4	0.003
5.6	20	1	5	3.5	1.5	0.006
10.0	20	11	55	(14.5)[a]	Aberrant Value	
18.0	20	7	35	38.0	3.0	0.004
32.0	20	12	60	67.0	7.0.	0.024
56.0	20	18	90	87.5	2.5	0.006
100.0	20	20	100 (99.0)[b]	97.0	2.0	0.014
					Total	0.057

a. Percent-affected organisms at the 10 percent effluent volume is obviously an aberrant value and should be omitted when fitting the line in Step 2.

b. Step 3 "Corrected" affected values from Table 7.

Step 4 (Cont.):

Calculation of Chi^2

a. Mean number of organisms used in 'K' (K=6) concentrations = $\frac{120}{6}$ = 20

(Note that the data for the 10% effluent volume were aberrent and not used. Therefore, K=6, and the total number of organisms = 120)

b. Calculated Chi^2 = 20 x 0.057 = 1.14

c. Degrees of Freedom (N) = K - 2 = 6 - 2 = 4

d. From Table 8, the Chi^2 for 4 degrees of freedom = 9.49

e. The calculated Chi^2 is less than the tabular Chi^2. Therefore, it is assumed the line is a good fit, and the data are non-heterogeneous.

Step 5:

a. From the fitted line in Fig. 2, determine the (percent) effluent concentrations corresponding to the 16%, 50% and 84% affected organism values:

b. LC84 effluent concentrations = 50.0%
LC50 " " = 23.0%
LC16 " " 10.5%

c. Calculate the slope function, 'S', as:

$$S = \frac{LC84/LC50 + LC50/LC16}{2} = \frac{50.0/23.0 + 23.0/10.5}{2}$$

$$= \frac{2.17 + 2.19}{2} = \frac{4.36}{2} = 2.18$$

d. N' = 40 (From Figure 2)

e. Calculate the exponent (N') and factor, f_{LC50}

$$f_{LC50} = S^{2.77/\sqrt{N'}} = 2.18^{2.77/\sqrt{40}} = 2.18^{2.77/6.32} = 2.18^{0.438} = 1.41$$

f. Calculate the confidence limits of the LC50

(1) Upper limit for 95% probability = LC50 X f_{LC50} = 23.0 X 1.4 = 32.2%

(2) Lower limit for 95% probability = LC50/f_{LC50} = 23.0/1.4 = 16.4%

TABLE 7. CORRECTED VALUES OF 0% OR 100% EFFECT

Expected Value	Corrected Value									
	0	1	2	3	4	5	6	7	8	9
0	--	0.3	0.7	1.0	1.3	1.6	2.0	2.3	2.6	2.9
10	3.2	3.5	3.8	4.1	4.4	4.7	4.9	5.2	5.5	5.7
20	6.0	6.2	6.5	6.7	7.0	7.2	7.4	7.0	7.8	8.1
30	8.3	8.4	8.6	8.8	9.0	9.2	9.3	9.4	9.6	9.8
40	9.9	10.0	10.1	10.2	10.3	10.4	10.4	10.4	10.4	10.5
50	--	89.5	89.6	89.6	89.6	89.7	89.7	89.8	89.9	90.0
60	90.1	90.2	90.4	90.5	90.7	90.8	91.0	91.2	91.4	91.6
70	91.7	91.9	92.2	92.4	92.6	92.8	93.0	93.3	93.5	93.8
80	94.0	94.3	94.5	94.8	95.1	95.3	95.6	95.9	96.2	96.5
90	96.8	97.1	97.4	97.7	98.0	98.4	98.7	99.0	99.3	99.7

TABLE 8. VALUES OF Chi^2 (p = 0.05)

Degrees of Freedom (N)	Chi^2
1	3.84
2	5.99
3	7.82
4	9.49
5	11.1
6	12.6
7	14.1
8	15.5
9	16.9
10	18.8

FIG. I. DATA SHEET FOR EFFLUENT TOXICITY TEST.

Industry/Toxicant _____
Address _____
Contact _____
Effluent Serial Number _____
NPDES Permit Number _____

Beginning: Date _____ Time _____
Ending: Date _____ Time _____
Test Organism _____
Test Temperature Range _____

Conc. or %	Test Container Number	Number of Live Organisms					Dissolved Oxygen (mg/l)					pH					Total Alkalinity (mg/l as $CaCO_3$)					Total Hardness (mg/l as $CaCO_3$)									
		0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96

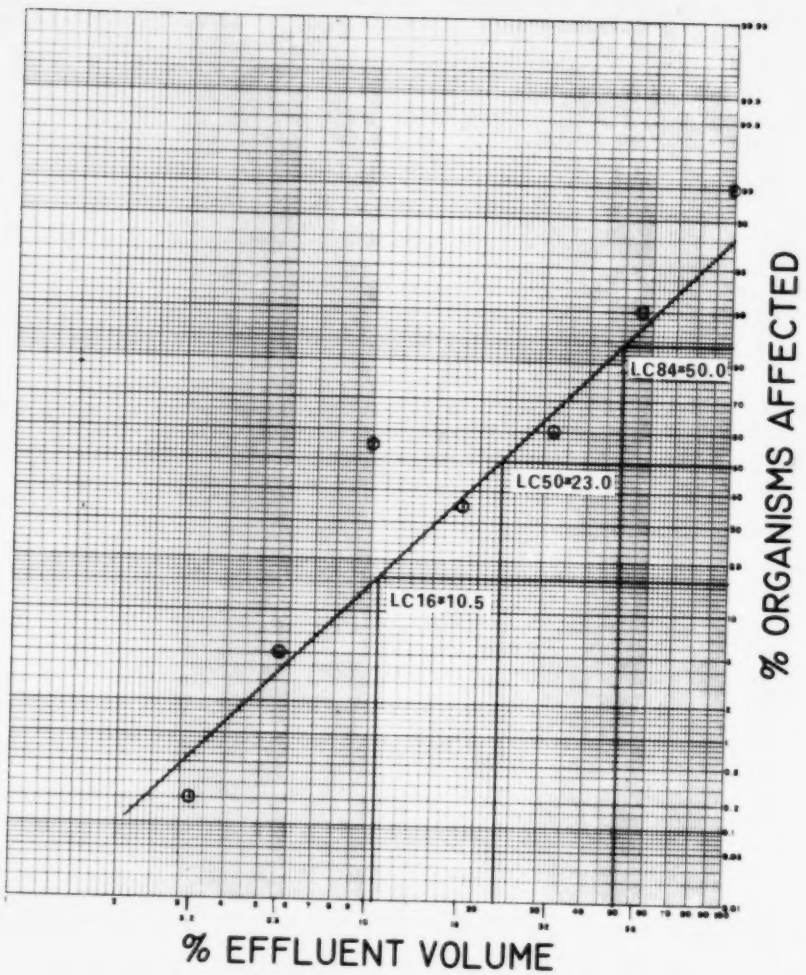


FIG. 2. LINE FITTED TO DATA, AND LC16, LC50, AND LC84 AS READ FROM THE LINE (STEPS 2, 3, AND 5).

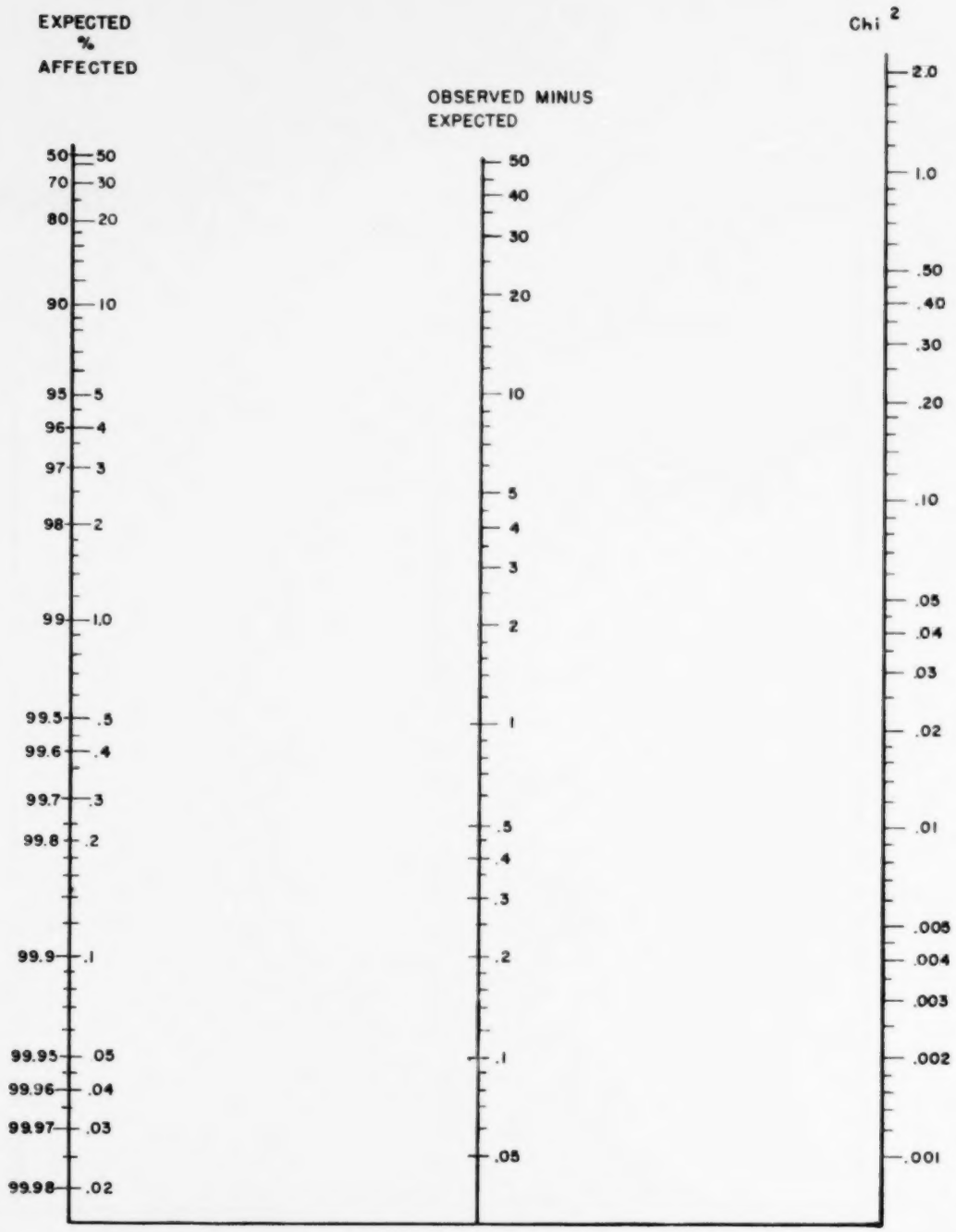


FIG. 3. NOMOGRAPH FOR OBTAINING Chi^2 FROM EXPECTED % AFFECTED AND OBSERVED–MINUS–EXPECTED (STEP 4).

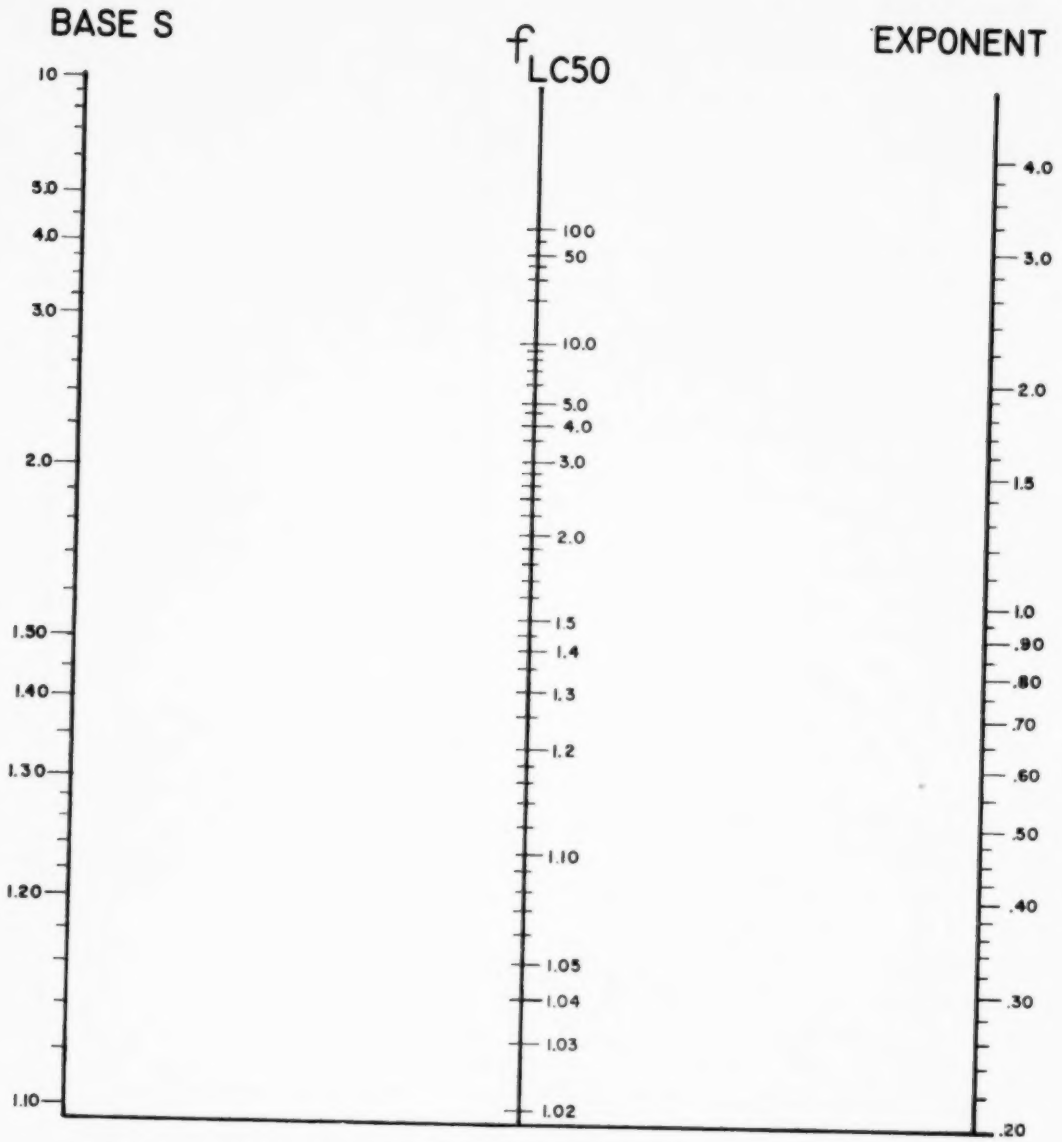


FIG. 4. NOMOGRAPH FOR RAISING BASE S TO A FRACTIONAL EXPONENT

B. LOG-CONCENTRATION VERSUS PERCENT-SURVIVAL METHOD OF DETERMINING THE LC50

General Procedure

Step 1: Plot the percent effluent volumes and the corresponding percent survival on semi-logarithmic paper (Fig. 5).

Step 2: Locate the 2 highest points on the graph which are separated by the 50 percent survival line and connect them with a diagonal straight line. However, if one of the points is an aberrant value, the next lowest or highest percent-effluent volume is used.

Step 3: Read on the scale for percent-effluent volume, the value of the point where the diagonal line and the 50 percent survival line intersect. This value is the LC50 percent-effluent volume for the test. If by chance one of the effluent concentrations happens to have 50 percent survival, no graphing is necessary.

Example

Step 1: The percent-effluent volumes and the corresponding percent survival data from the Litchfield and Wilcoxon example are plotted in Fig. 5.

Step 2: The two highest points which are separated by the 50 percent survival line (65 percent and 40 percent) are located and connected with a diagonal diagonal straight line. The percent survival in the 10 percent-effluent volume was considered an aberrant value and, therefore, was omitted from the evaluation.

Step 3: An LC50 of 25.4 percent-effluent volume for the test was derived from the point where the diagonal line and the 50 percent survival line intersected in Fig.5.

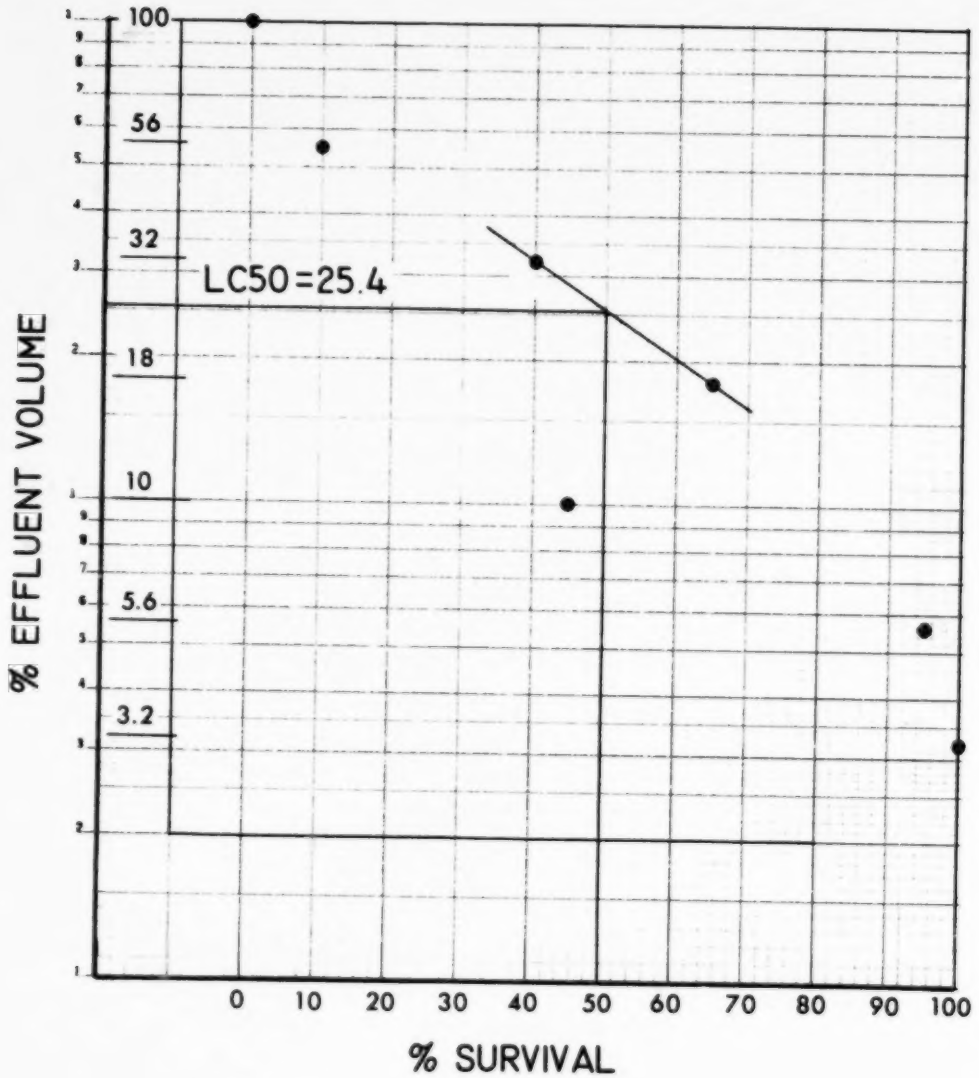


FIG. 5. PLOTTED DATA AND FITTED LINE FOR LOG–CONCENTRATION VERSUS % SURVIVAL METHOD

C. DILUTOR SYSTEMS, CONTROL PANEL AND EQUIPMENT LISTS

Two proportional dilutor systems are illustrated: (1) The Solenoid Valve System, and (2) the Vacuum Siphon System. The designs incorporate features from devices developed by many other Federal and State programs, and have been shown to be very versatile for on-site bioassays in mobile labs as well as in fixed (central) labs. The Solenoid Valve System is fully controlled by solenoids (Figs. 6, 7, & 8), and is the preferred system. The Vacuum Siphon System (Figs. 6, 9, & 10), however is acceptable. Both systems employ the same control panel (Fig. 14).

If in the range-finding test, the LC50 of the effluent falls in the concentration range, 5.6% to 100%, premixing is not required, and the mixing chamber is by-passed by running a TEFLON^{R} tube directly from the effluent in-flow pipe to chamber E-1. Chambers D-1 and D-2 and the mixing chamber are deactivated.

To provide the capability of using the dilutor system to carry out tests of the toxicity of pure compounds, the control panel is equipped with an auxillary power receptacle to operate a metering pump to deliver an aliquot of the stock solution of the pure compound directly to the mixing chamber during each cycle. In this case, chamber D-1 is deactivated and chamber D-2 is calibrated to deliver a volume of 2000 ml, which is used to dilute the aliquot to the highest concentration used in the toxicity test.

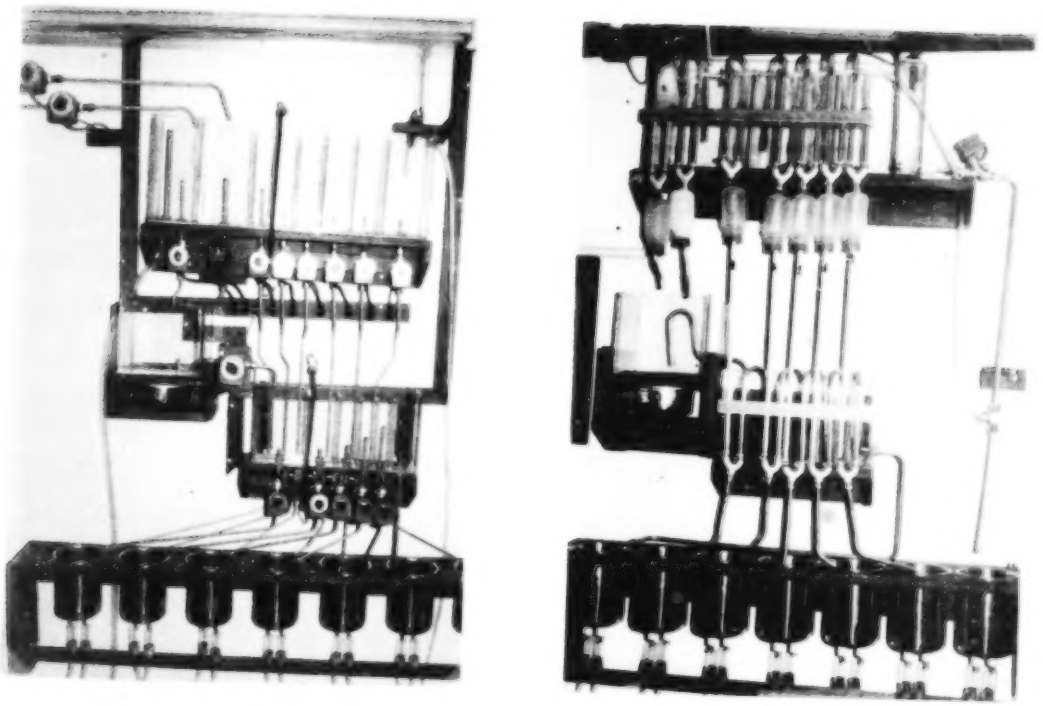


FIG. 6. PHOTOGRAPHS OF DILUTOR SYSTEMS: SOLENOID VALVE SYSTEM (LEFT), AND VACUUM SIPHON SYSTEM (RIGHT).

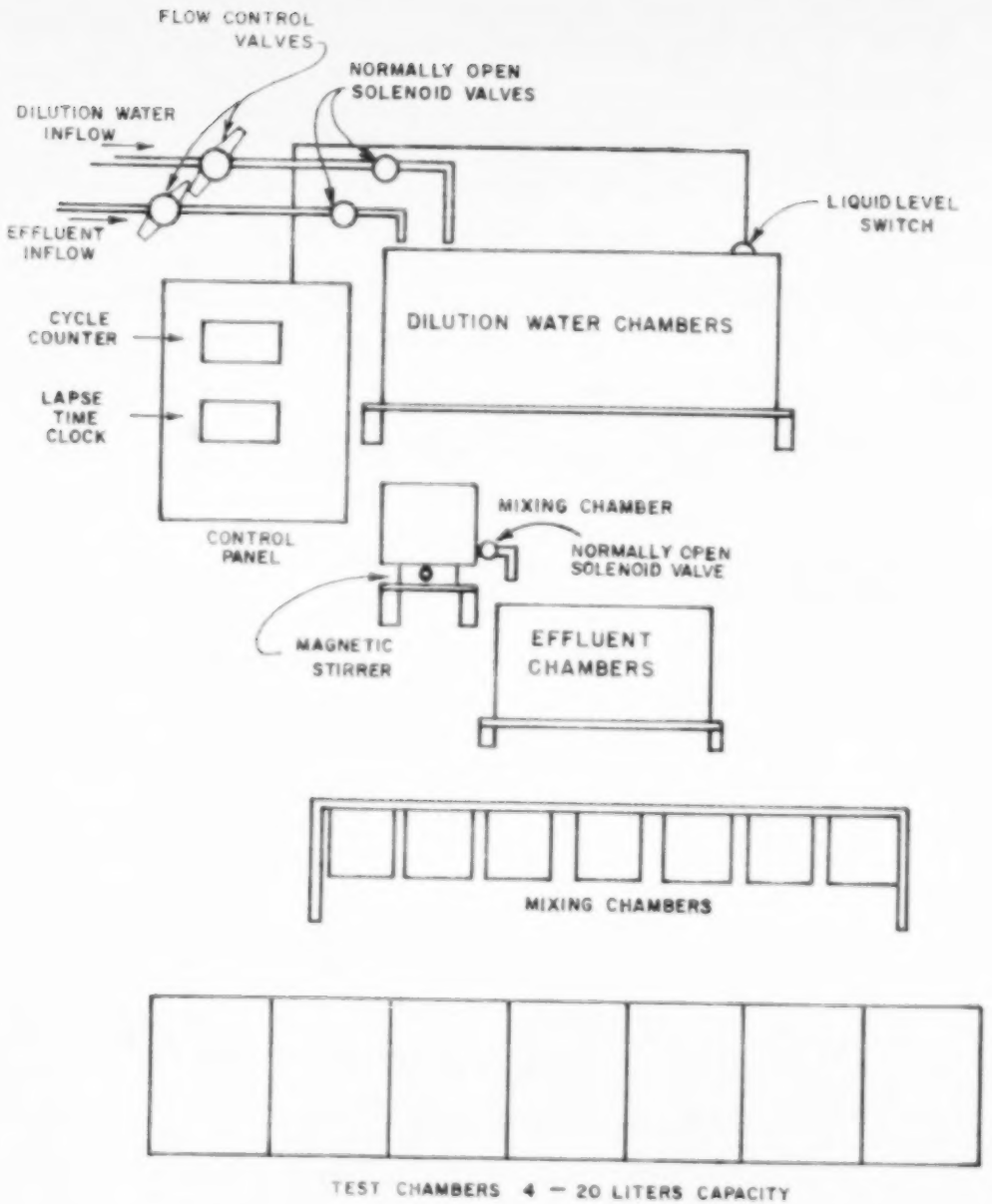


FIG. 7. SOLENOID VALVE DILUTOR SYSTEM, GENERAL DIAGRAM.

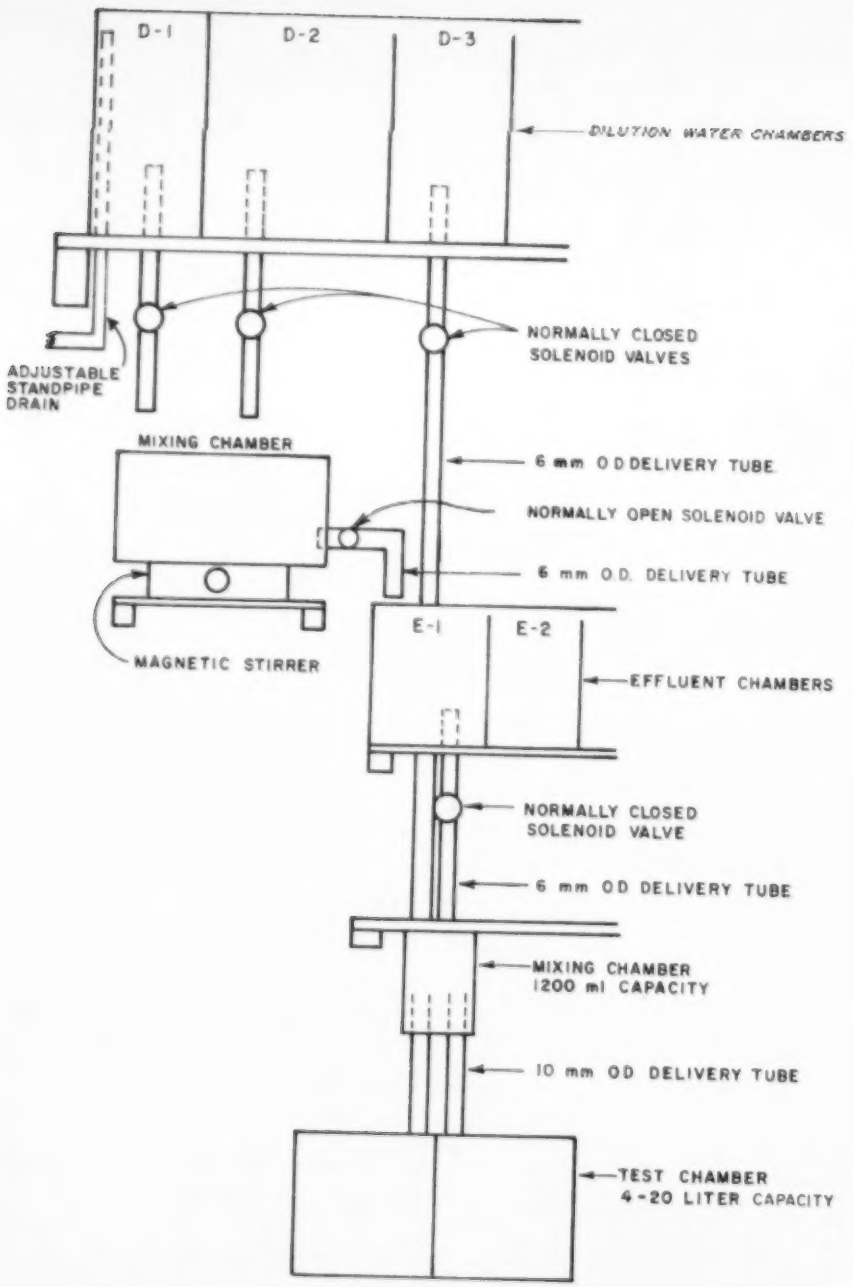


FIG. 8. SOLENOID VALVE DILUTOR SYSTEM, DETAILED DIAGRAM.

Solenoid System Equipment List

1. Diluter Glass.
2. Stainless Steel Solenoid Valves
   a. 3 - normally open, two-way, 55 psi, water, 1/4" pipe size, 9/32" orifice size, ASCO 8262152, for incoming effluent and dilution water pipes and mixing chamber pipe.
   b. 1 - normally closed, two-way, 15 psi, water, 3/8" pipe size, 3/8" orifice size, ASCO 8030B65, for D-2 chamber evacuation pipe.
   c. 12 - normally closed, two-way, 36 psi, water, 1/4" pipe size, 9/32" orifice size. ASCO 8262C38, for remaining dilution chamber and effluent chamber evacuation pipes.
3. Stainless steel tubing, seamless, 316-grade, austenetic.
   a. 10 ft - 3/8" OD, 0.035" wall thickness, for dilution water and effluent pipes.
   b. 60 ft - 1/4" OD, 0.035" wall thickness, for dilution water and effluent pipes.
   c. 1 ft - 3/4" OD, 0.035" wall thickness, for standpipe in D-1 chamber.
4. Swagelok tube connectors, stainless steel.
   a. 4 - male tube connectors, male pipe size 1/4", tube OD 3/8".
   b. 2 - male tube connectors, male pipe size 1/2", tube OD 3/8".
   c. 26 - male tube connectors, male pipe size 1/4, tube OD 1/4"
   d. 2 - male tube connectors, male pipe size 3/8", tube OD 3/8".
   e. 2 - male adapter, tube to pipe, male size 1/2", tube OD 3/8".
5. 7 - 1200 ml stainless steel beakers.
6. Several lbs each of Neoprene stoppers, size 00, 0, and 1, 1 lb of siz 5.
7.]4 - aquarium 2-5 gal.
8. Magnetic stirrer.
9. 2 - PVC ball valves, 1/2" pipe size.
10. Dilutor control panel - see Fig. 14 and equipment list.
11. Plywood sheeting, exterior grade: one - 4' x 8' x 3/4", one - 4' x 8' x 1/2".
12. Pine or redwood board, 1" x 8", 20 ft.
13. Epoxy paint, 1 gal.
14. Assorted wood screws, nails, etc.
15. 25 ft - 1/4" ID, TEFLON^{R} tubing, to connect the mixing chambers to the test chambers (see Fig. 10).

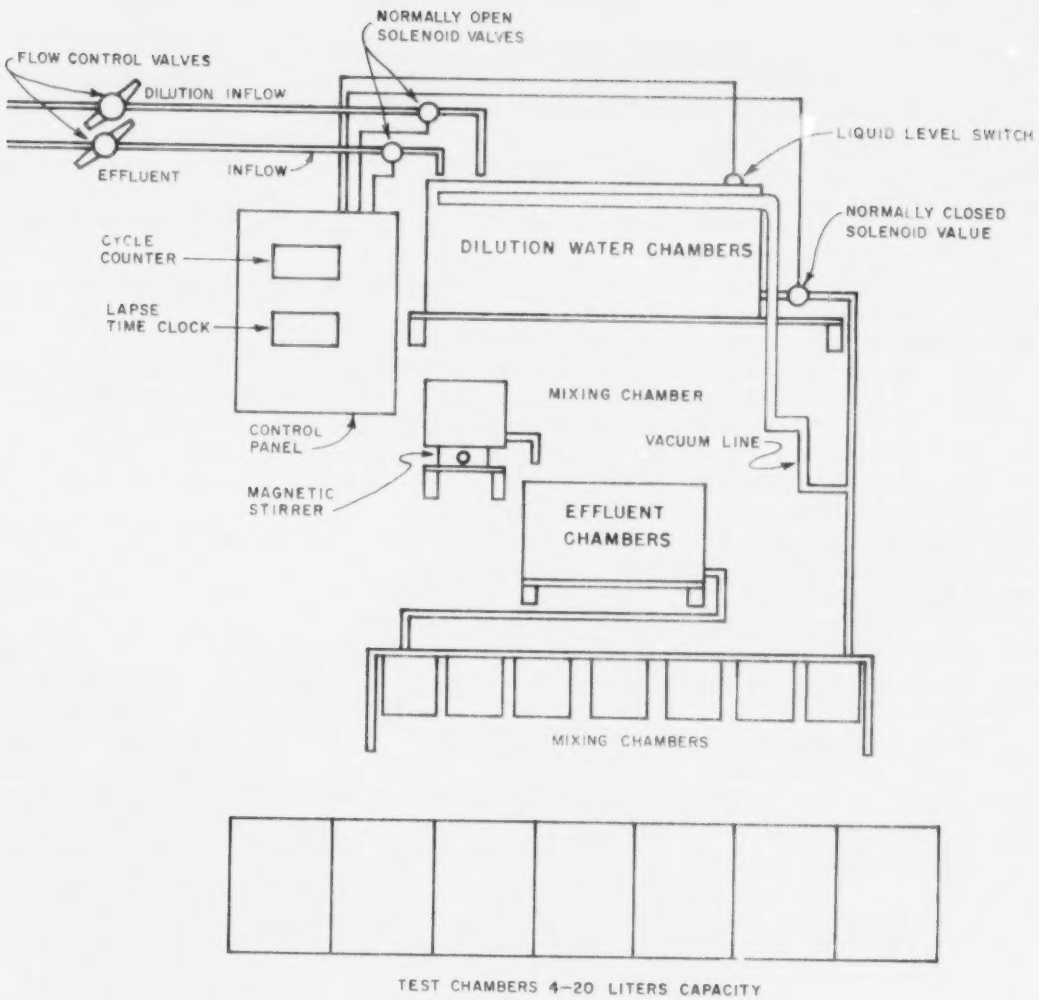


FIG. 9. VACUUM SIPHON DILUTOR SYSTEM, GENERAL DIAGRAM.

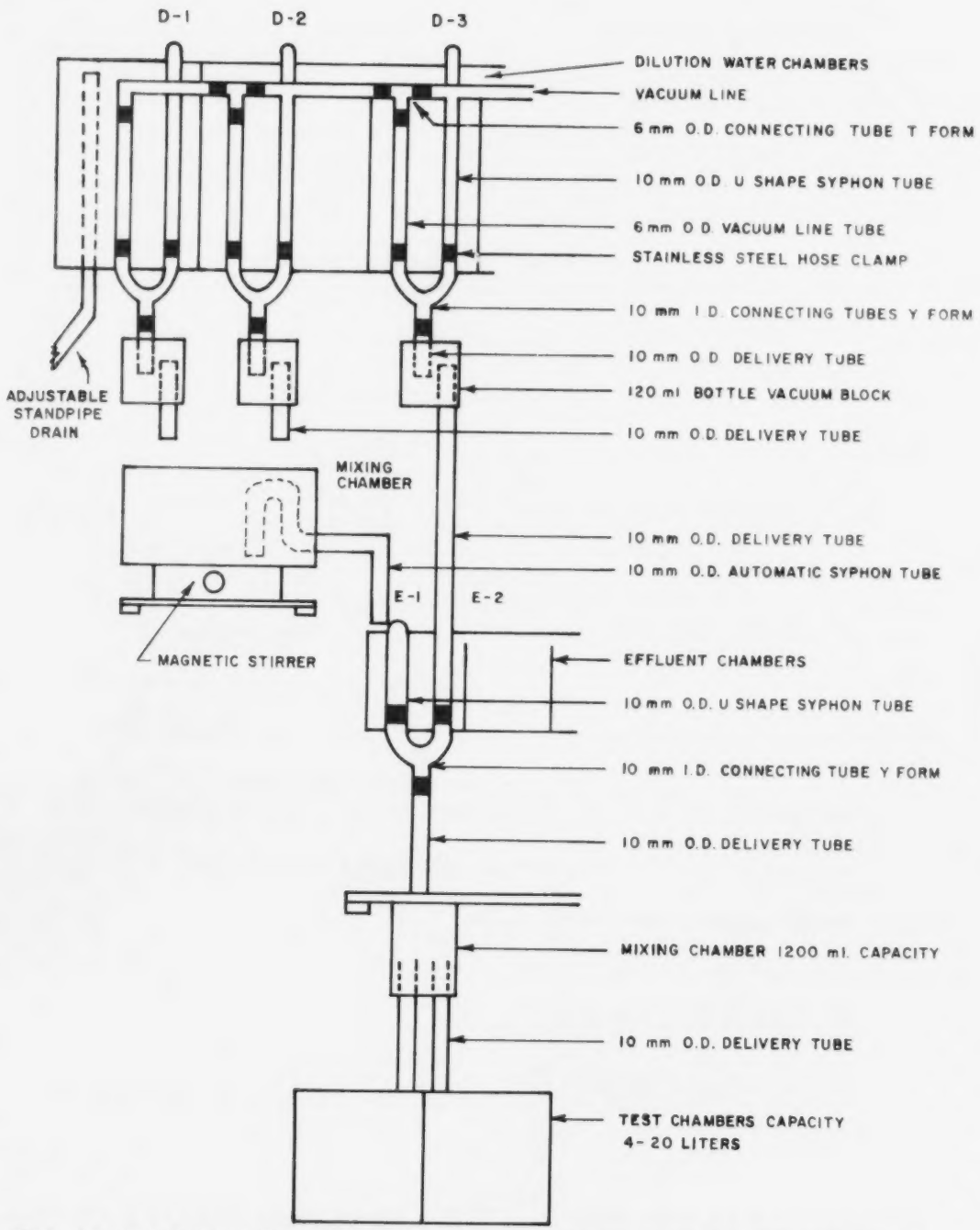
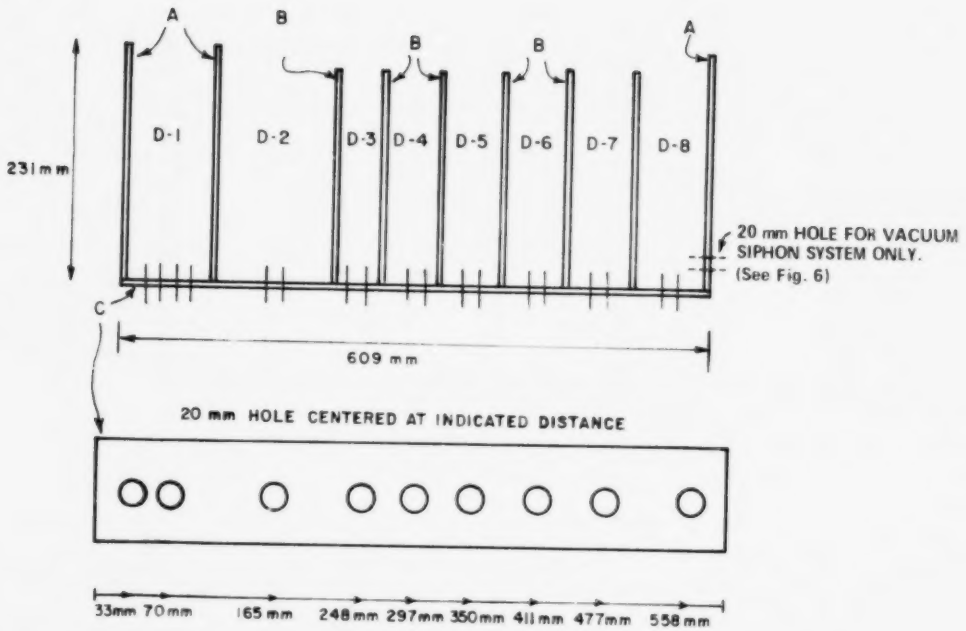

D-1
D-2
D-3
DILUTION WATER CHAMBERS
VACUUM LINE
6 mm O.D. CONNECTING TUBE T FORM
10 mm O.D. U SHAPE SYPHON TUBE
6 mm O.D. VACUUM LINE TUBE
STAINLESS STEEL HOSE CLAMP
10 mm I.D. CONNECTING TUBES Y FORM
10 mm O.D. DELIVERY TUBE
120 ml BOTTLE VACUUM BLOCK
10 mm O.D. DELIVERY TUBE
ADJUSTABLE STANDPIPE DRAIN
MIXING CHAMBER
10 mm O.D. DELIVERY TUBE
10 mm O.D. AUTOMATIC SYPHON TUBE
E-1
E-2
MAGNETIC STIRRER
EFFLUENT CHAMBERS
10 mm O.D. U SHAPE SYPHON TUBE
10 mm I.D. CONNECTING TUBE Y FORM
10 mm O.D. DELIVERY TUBE
MIXING CHAMBER 1200 ml. CAPACITY
10 mm O.D. DELIVERY TUBE
TEST CHAMBERS CAPACITY 4-20 LITERS

FIG. 10. VACUUM SIPHON DILUTOR SYSTEM, DETAILED DIAGRAM.

Vacuum Siphon System Equipment List

1. Diluter Glass.
2. Stainless steel solenoid valves.
   a. 2 - normally open, two-way, 55 psi, water, 1/4" pipe size, 9/32" orifice size, ASCO 8262152, for incoming effluent and dilution water pipes.
   b. 1 - normally closed, two-way, 15 psi, water, 3/8" pipe size, 3/8" orifice size, ASCO 8030B65, for dilution water evacuation pipe.
3. Stainless steel tubing, seamless, 316-grade, austenetic.
   a. 60 ft - 3/8" OD, 0.035" wall thickness, for dilution water and effluent pipes.
   b. 20 ft - 5/16" OD, 0.035" wall thickness. for standpipes in mixing chambers.
   c. 1 ft - 3/4" OD, 0.035" wall thickness, for standpipe in D-1 chamber.
4. Swagelok tube connectors, stainless steel
   a. 4 - male tube connectors, male pipe size 1/4", tube OD 3/8".
   b. 2 - male tube connectors, male pipe size 3/8", tube OD 3/8".
   c. 2 - male adapter, tube to pipe, male pipe size 1/2", tube OD 3/8".
   d. 2 - male tube connectors, male pipe size 1/2", tube OD 3/8".
5. 7 - 1,200 ml stainless steel beakers.
6. Several lbs each of NEOPRENE^{R} stoppers, size 00, 0 and 1; 1 lb of size 5.
7. 14 - aquariums, 2-5 gal.
8. Magnetic stirrer.
9. 2 - PVC Ball valves, 1/2" pipe size.
10. Dilutor control panel equipment - see Fig. 14 and equipment list.
11. 7 - 120 ml NALGENE^{R} bottles.
12. 3 ft, 1-in-2 aluminum bar, for siphon support brackets.
13. Stainless steel set screws, box of 50, for securing SS tubing in siphon support brackets.
14. Stainless steel hose clamps, box of 10, size #4 or 5,(need 3 boxes).
15. 6 - NALGENE^{R} T's, 5/16" OD.
16. 12 - TYGON^{R} Y connectors, 3/8" I.D.
17. TYGON^{R} tubing, 3/8" OD, 10 ft.
18. Plywood sheeting, exterior grade: one - 4' x 8x x 3/4", one - 4' x 8' x 1/2".
19. Pine or redwood board, 1" x 8", 20 ft.
20. Epoxy paint, 1 gal.
21. Assorted wood screws, nails, etc.
22. 25 ft - 5/16" ID, TEFLON^{R} tubing, to connect the mixing chambers to the test chambers.



DRAIN HOLES IN BOTTOM PLATE (C) SHOWN FOR SOLENOID VALVE DILUTOR SYSTEM. FOR VACUUM SIPHON DILUTOR SYSTEM, SINGLE DRAIN HOLE IS REQUIRED ONLY FOR CHAMBER D-1.

INDIVIDUAL PART SIZE AND NUMBER OF PIECES USING 6 mm (1/4") PLATE GLASS

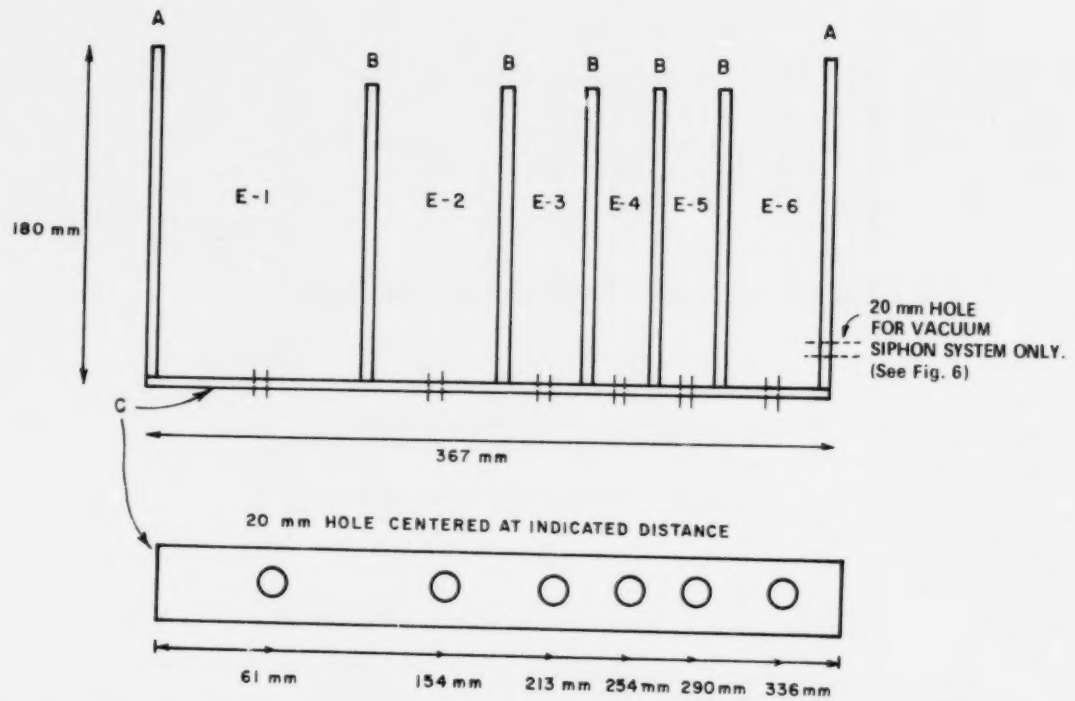
A 225 mm X 95 mm - 3
B 200 mm X 95 mm - 6
C 609 mm X 95 mm - 1 (Bottom Plate)
D 609 mm X 231 mm - 2 (Side Panels)

INSIDE CELL MEASUREMENTS AND APPROXIMATE VOLUME

D - 1 95 mm X 225 mm X 95 mm - 2030 ml
D - 2 115 mm X 200 mm X 95 mm - 2185 ml
D - 3 40 mm X 200 mm X 95 mm - 760 ml
D - 4 45 mm X 200 mm X 95 mm - 855 ml
D - 5 50 mm X 200 mm X 95 mm - 950 ml
D - 6 60 mm X 200 mm X 95 mm - 1140 ml
D - 7 60 mm X 200 mm X 95 mm - 1140 ml
D - 8 90 mm X 200 mm X 95 mm - 1710 ml

NOTE: 1/8" - 316 GRADE AUSTENISTIC STAINLESS STEEL MAY BE SUBSTITUTED FOR GLASS IN PART C.

FIG. II. DILUTION WATER CHAMBERS



DRAIN HOLES IN BOTTOM PLATE (C) SHOWN FOR SOLENOID VALVE DILUTOR SYSTEM. FOR VACUUM SIPHON DILUTOR SYSTEM, SINGLE DRAIN HOLE IS REQUIRED ONLY FOR CHAMBER D-1.

INDIVIDUAL PART SIZE AND NUMBER OF PIECES USING 6mm (1/4") PLATE GLASS

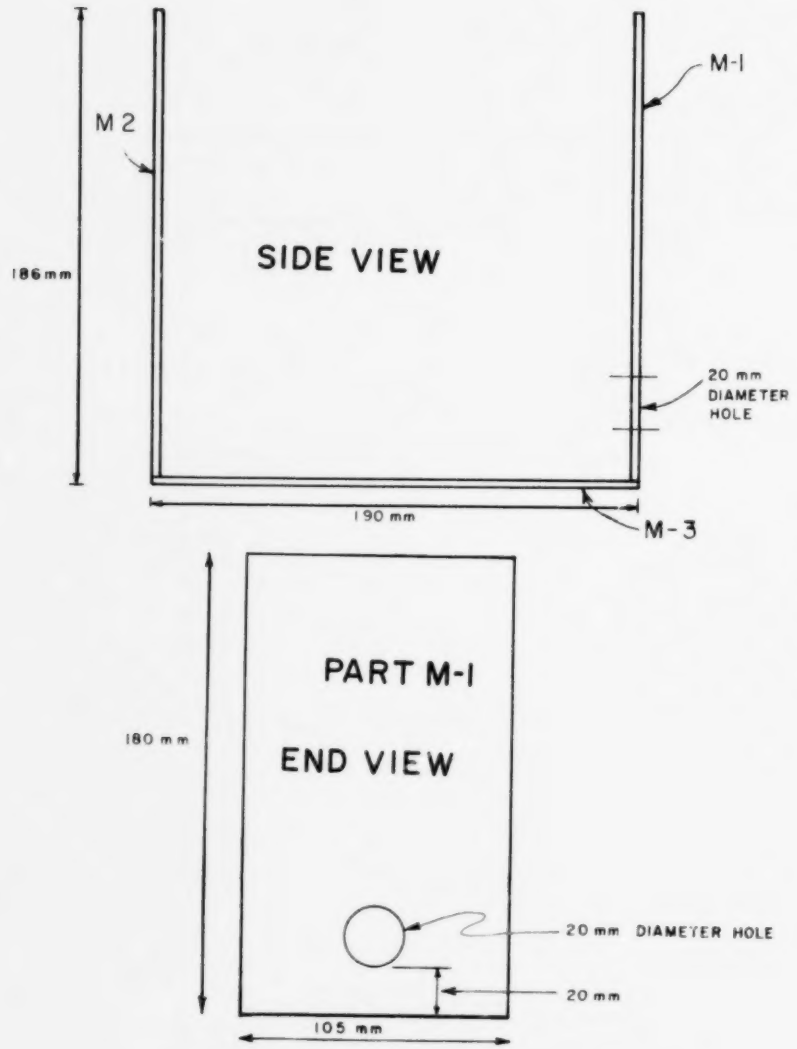
A 174 mm X 40 mm - 2
B 155 mm X 40 mm - 5
C 367 mm X 40 mm - 1 (Bottom Plate)
D 367 mm X 180 mm - 2 (Side Panels)

INSIDE CELL MEASUREMENTS AND APPROXIMATE VOLUME

E-1 110 mm X 155 mm X 40 mm - 682 ml
E-2 65 mm X 155 mm X 40 mm - 403 ml
E-3 40 mm X 155 mm X 40 mm - 248 ml
E-4 30 mm X 155 mm X 40 mm - 186 ml
E-5 30 mm X 155 mm X 40 mm - 186 ml
E-6 50 mm X 155 mm X 40 mm - 318 ml

NOTE: 1/8" - 316 GRADE AUSTENISTIC STAINLESS STEEL MAY BE SUBSTITUTED FOR GLASS IN PART C.

FIG. 12. EFFLUENT CHAMBERS



INDIVIDUAL PART SIZE AND NUMBER OF PIECES USING 6mm (1/4") PLATE GLASS

M-1 180mm X 105mm-1
M-2 180mm X 105mm-1
M-3 190mm X 105mm-1 (BOTTOM PLATE)
M-4 190mm X 186mm-2 (SIDE PANELS)

(APPROXIMATE CAPACITY 3365 ml)

FIG. 13. MIXING CHAMBER

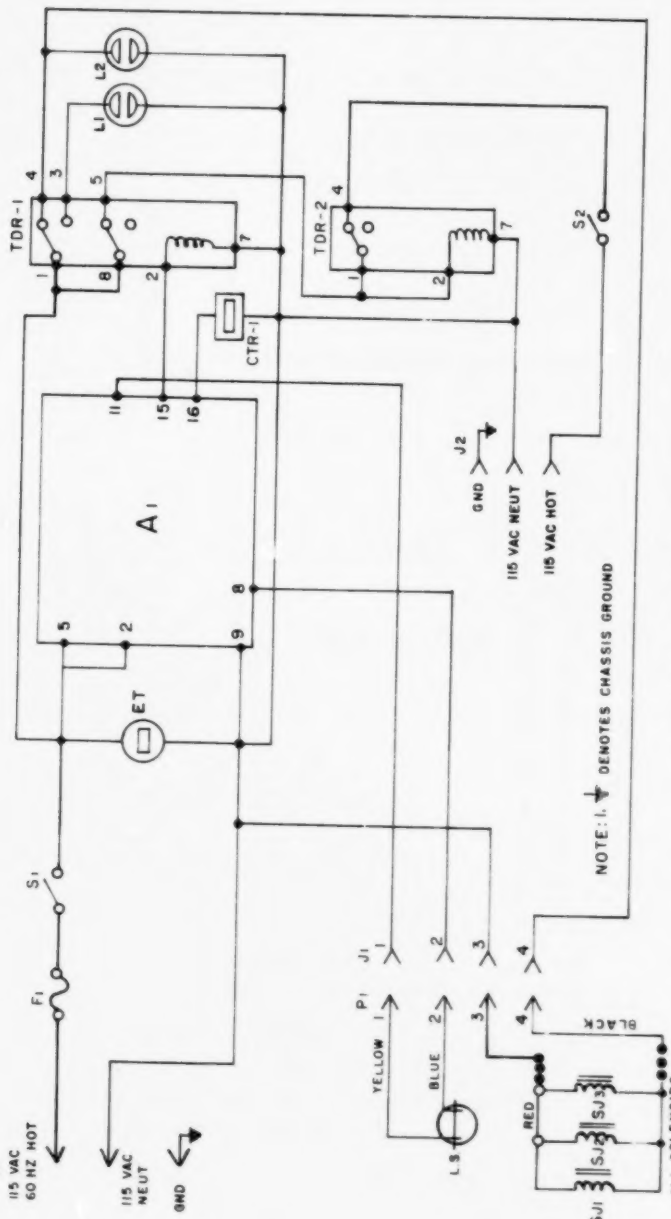


FIG. 14. DILUTOR CONTROL PANEL.

Dilutor Control Panel Equipment List*

Designation	CKT Description	Manufacturer
A_1	Encapsulated amplifier	Cutler Hammer 1353H98C
CTR-1	Cycle counter	Rodington #P2-1006
ET	Elapsed time indicator	Courac #636W-AA H&T
F_1	Input power fuse	Little fuse 342038
J_1	Recepticle	Amphenol 91PC4F
J_2	Aux A.C. output jack	Stand. 3-prong AC Recpt.
J_3	Main input power cord	Stand. 3-prong AC maleplug
L_1	Fill indicator light	Dialco 95-0408-09-241
L_2	Emptying indicator light	Dialco 95-0408-09-241
L.S.	Level sensor (Dual Sensing Probe)	Cutler Hammer 13653H2
P_1	Plug	Amphenol 91MC4M
S_1	On-off main power switch (spst)	Cutler Hammer 7580 K7
S_2	On-off aux power switch (spst)	Cutler Hammer 7580 K7
SJ_1	Solenoid	(See Solenoid and Vacuum System equipment lists)
SJ_2	"	" " "
SJ_3	"	" " "
SJ_4 - SJ_{16}	Additional Solenoids for Solenoid Valve System	" " "
TDR-1	Time delay relay	Dayton 5x829
TDR-2	Aux time delay relay	Dayton 5x829

*Consult local electric supply house.